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# COMPETITIVE HYBRIDIZATION TO DETECT RNA SPECIES IN BRAIN INDUCED DURING LEARNING

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# COMPETITIVE HYBRIDIZATION TO DETECT RNA SPECIES IN BRAIN INDUCED DURING LEARNING

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Running Title:

COMPETITIVE HYBRIDIZATION WITH BRAIN RNA

# COMPETITIVE HYBRIDIZATION TO DETECT RNA SPECIES IN BRAIN INDUCED DURING LEARNING

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## INTRODUCTION

There is now considerable circumstantial evidence to implicate protein[1,19,3] and RNA[43,24] synthesis in memory consolidation, and one is encouraged to assume the working hypothesis that neurons operate during learning by the same basic cellular mechanisms believed to be involved in cellular development and differentiation. That is, electrical stimuli reaching the brain will act through inducer substances to derepress genes, giving rise to messenger RNA molecules which effect the synthesis of proteins. These proteins, acting as enzymes or structural units, will then cause a relatively permanent alteration in the electrical circuitry of the brain. The time required for the development of long-term memory is sufficient to allow the induction of RNA and the synthesis of proteins. Such a derepression model has been advocated by Bonner[8], who proposed a test for this model. Since these new RNA molecules would be gene products and present in learning animals, but not in non-learning animals, they should be able to be pulse labeled and detected by competitive hybridization experiments.

Messenger RNA molecules are synthesized in cell nuclei on DNA so that they have base sequences complementary to those of the DNA segments coding for those RNA molecules. Hybridization refers to the *in vitro* recombination of isolated nucleic acids with complementary base sequences. The extent of RNA-DNA hybridization is usually measured by

radioactive labeling of the RNA and determination of the fraction of the radioactivity that becomes attached to the DNA when the two are incubated together under appropriate conditions. In competitive hybridization, non-labeled RNA is added to the labeled RNA, or hybridized with the DNA before the labeled RNA, and the amount of base sequence identity between the RNA's is estimated by the extent to which the non-labeled RNA competes for the sites to which the labeled RNA would hybridize.

The "detection of RNA species unique to a behavioral task" by competitive hybridization has recently been reported by Machlus and Gaito[27,28]. A description of their work will illustrate the method. In their experiment, DNA and RNA were isolated from the brains of trained and naive rats. For labeled RNA, animals were sacrificed 90 minutes after intracranial injection of 200 μc of $^{3}H$-orotic acid. Animals to be trained were placed in a training box 60 minutes after injection (unlabeled animals were injected with unlabeled orotic acid). After 15 minutes of adaptation to the training apparatus, they were given 15 minutes of shock avoidance training. The animals were sacrificed immediately thereafter, and RNA and DNA were isolated separately by phenol extraction. Fifty μg of DNA was hybridized for 12 hours with 50 μg of competitor RNA and then 12 hours with 50 μg of labeled testor RNA. Hybridization was carried out by the method of Gillespie and Spiegelman[20]. The essential data are the following:

| | % Labeled RNA Hybridized |
|---|---|
| $DNA_L - RNA_L^*$ | 3.10 ± .24 |
| $DNA_L - RNA_{NL} - RNA_L^*$ | 1.40 ± .21 |
| $DNA_L - RNA_L - RNA_{NL}^*$ | 0 ± 0 |

Subscripts L and NL refer to DNA or RNA from learning and non-learning animals respectively; * means RNA is labeled.

Preincubation of DNA with RNA from non-learning animals inhibited hybridization of the DNA with labeled RNA from learning animals 55%, while preincubation with RNA from learning animals inhibited hybridization with labeled RNA from non-learning animals 100%. Since $RNA_{NL}$ did not mask all the DNA sites for hybridization with complementary $RNA_L$*, it was inferred that new species of RNA were present in the RNA from the trained animals. The fact that RNA from learning animals ($RNA_L$) masked all the sites for RNA from non-learning animals ($RNA_{NL}$*) was considered additional support for this view.

Most researchers interested in molecular mechanisms in the brain as related to learning are not experienced with the technique of competitive hybridization, and may have some difficulty in evaluating these results. Because of the fundamental importance of this type of experiment to our understanding of molecular brain mechanisms, I have attempted to reproduce these results, and provide further controls on the method. The discussion section refers to a number of other articles relevant to the interpretation of competitive hybridization data.

## MATERIALS AND METHODS

### Labeling

Sprague-Dawley male rats, 200 - 250 grams, were used in all experiments. The rats were injected intraventricularly with 40 μc 5-$^3$H-uridine (New England Nuclear, 20 C/mM) in physiological saline, 20 μl on each side. Unlabeled animals were injected with the same volume of physiological saline.

### Training

Rats were put in the training apparatus 60 minutes after injection, and, after 15 minutes of adaptation to the apparatus, were trained for

15 minutes. Shock avoidance training consisted of teaching the rats to run from a dark box (for which they have a natural preference) through an opening into a lighted box. If the animal did not escape to the lighted box within 10 seconds of being placed in the dark box, a bell was sounded for 2 seconds followed by a shock for 2 seconds; the bell and shock were repeated every 10 seconds until the animal escaped to the lighted box, where it was allowed to remain until the next trial. For each trial the animal was returned to the dark box, and the escape latency recorded. All trained animals received 15 trials in 15 minutes.

Nucleic acid isolation

DNA was isolated by the method of Marmur[29] from a crude nuclear fraction from rat liver. The rats were starved for 24 hours before sacrifice for DNA isolation, and a pronase step was included in the procedure immediately following the RNase step.

The following is the standard procedure used for RNA isolation: A 10% homogenate of whole brain, including cerebellum and olfactory bulbs, is made in cold 0.5% naphthalene disulfonate containing bentonite. Less than 1 minute elapsed between decapitation and homogenization. This is extracted immediately with an equal volume of phenol II (phenol, water, m-cresol and 8-hydroxyquinoline, 500:55:70:0.5) at 60°C for 15 minutes. The solution is rapidly and thoroughly chilled on ice and centrifuged in the Spinco SW-25 rotor at 20,000 rpm for 5 minutes. The aqueous layer is added to 1/10 volume of 1% sodium dodecylsulfate (SDS) plus bentonite. The mixture is reextracted at room temperature for 5 minutes and chilled and centrifuged as before, once with phenol II and once with chloroform. SDS and bentonite are added to each aqueous

layer except the last. The final aqueous layer is made 0.1 M NaCl, 2 volumes of ethanol are added and the RNA is precipitated overnight at -20°C. The RNA is collected by centrifuging at 1,000 g for 10 minutes, dissolved in water and reprecipitated with NaCl and ethanol. The typical yield of RNA was approximately 2.0 mg per brain based on the optical density at 260 mμ (1 mg = 25 O.D. units). Sucrose density gradients of RNA isolated by this procedure showed no detectable breakdown of the RNA.

Hybridization

Hybridization was carried out by the method of Gillespie and Spiegelman[20]. DNA was immobilized on nitrocellulose membranes which, after being dried, were incubated with RNA in 1 ml of 6 X SSC (1 X SSC = .15 M sodium chloride, .015 M sodium citrate, pH 7.0) at 66°C in scintillation vials. In competition experiments membranes were hybridized for 12 hours with competitor RNA, at which time testor RNA was added and the hybridization continued for 12 hours. For the determination of total hybrid, membranes at the conclusion of hybridization were rinsed in 3 washes of 2 X SSC, dried and the radioactivity remaining on the membranes determined by scintillation counting. For the determination of RNase resistant hybrid, membranes were incubated with pancreatic RNase (previously heated to 95°C for 10 minutes) for 1 hour at room temperature at a concentration of 20 μg/ml. The activity on membranes containing no DNA which were incubated with labeled RNA and then washed as usual was indistinguishable from background. Input activities were determined by applying 50 μg of RNA directly onto a membrane, drying the membrane and counting.

RESULTS

Learning is clearly evident with the training procedure used in these experiments. Figure 1 shows that the rats on the average learn to escape without receiving a shock after about 5 trials. After about 10 trials they run immediately from the dark box to the lighted box.

The results of our experiments comparing RNA from trained and naive rats as competitor and testor are shown in Table I. In the first two pairs of data, we see the results when different amounts of RNA, prepared individually from 4 trained and 4 naive rats, are tested for their effectiveness as competitor in the hybridization of pooled, labeled RNA from 4 trained rats. The figures for percent inhibition are the average of 4 determinations, using competitor RNA from 4 different rats. Percent inhibition, which indicates the extent of competition, is calculated from the level of hybridization of testor when no competitor was present during the preincubation period. The specific activity of testor RNA was about 30 cpm/μg, and the level of hybridization without compeitor was about 2.5% for total hybrid and 1.0% for RNase resistant hybrid.. The inhibition levels in Table I were calculated from total hybrid, because the activity in RNase resistant hybrid was so low. No statistically significant difference between the trained and naive RNA is observed. It should be noted that even with 200 μg of competitor only about 55% inhibition was observed.

In the third pair of data in Table I, trained and naive RNA are compared as testor. New RNA was prepared for this experiment. Competitor RNA from each of 4 naive rats was tested against testor from each of 4 trained and 4 naive rats, so that each percent inhibition is the

average of 16 determinations. In this experiment, as in the first experiment, no difference between trained and naive RNA was observed. The competition levels were the same as in that part of the first experiment where the same amount of competitor was used.

The moderate amount of competition in these experiments is in marked contrast to the complete competition reported by Machlus and Gaito for RNA from trained rats. To examine further the question of how much competition should be expected, a third experiment was performed using various amounts of trained competitor and a fixed amount of testor. Figure 2 shows the results of this experiment. Each point is the average of duplicate determinations. Fluctuation in the data is considerable, but it is clear that there is only about 50% inhibition with 200 μg of competitor. The figure also shows that inhibition levels can be calculated equally well from total or RNase resistant hybrid. The inhibition levels in this experiment are comparable to those in Table I.

Two of the factors which help explain why complete inhibition is not achieved can be appreciated from the data in Figures 3 and 4. Increasing amounts of labeled RNA were hybridized for 12 hours with 50 μg of DNA. In the region of 50 to 200 μg of RNA, there is a nearly linear increase in the amount of hybrid formed (Figure 3). This means that in this region the DNA sites complementary to the labeled RNA are not saturated, and hence complete inhibition in the competition experiments could not be expected. Figure 4 shows the effect of increasing the duration of the hybridization. There is an increase in the extent

of hybridization when the incubation is extended to 24 and 36 hours, indicating that the reaction had not gone to completion after 12 hours, and, again, saturation was not achieved with the 12-hour incubation. In all these experiments RNase resistant hybrid was about 40% of the total hybrid.

## DISCUSSION

All possible combinations of competitor and testor are represented in Table II. Naive and trained RNA can be compared as competitor, A versus B, or as testor, A versus C. In the first case, competition should be greater in B than in A if induction has taken place, since the trained competitor in B will have species in common with the testor which are absent in A. In the second case, competition should be greater in C than A, since in C, as in B, the composition of the competitor and testor should be identical. B and D (or C and D) need not be compared, since in B, C and D the competitor should contain all the species present in the testor.

Machlus and Gaito have looked at A and D in Table II. From the lack of complete competition in A, they inferred that new RNA species were present in the RNA from the brains of the trained animals. They did not look at B or C as a control. Our data with B and C, and the data of others to be discussed later, indicate that complete competition should not have been expected. With D they observed complete competition, no activity on the membranes after hybridization with testor. We observe with D, using the same amounts of DNA, competitor RNA and testor RNA as Machlus and Gaito, the same level of competition as we do in A, B and C, about 35%.

The results in this report have failed to show any differences between the hybridization characteristics of RNA extracted from the brains of trained and naive rats. Let us look at the systems in which hybridization has been used to detect induction or differences in RNA populations, and then examine in more detail the characteristics of various hybridization systems in order to evaluate the potential use of this technique for studying the relationship between learning and macromolecular metabolism in the brain.

In the early days of the messenger RNA theory, hybridization was used in elegant experiments with bacterial systems by Attardi et al.[2] and Hayashi et al.[22] to demonstrate the induction of specific messenger RNA's by inducer substances. Special techniques were used in these bacterial systems to increase greatly the sensitivity of the method. For instance, "non-relevant" m-RNA was removed by hybridization with DNA from mutants lacking the genes under study, and the relative concentration of the DNA segment carrying the genes under study was increased by using DNA from transducing phages such as λdg in which specific bacterial genes were incorporated into the episome by recombination. Also in this work very high specific activities were used.

In spite of the fact that these special techniques cannot be used with higher organisms, it has been possible to demonstrate differences in the RNA's synthesized by animal cells during periods of major alteration of structure or function. Competitive hybridization has been used by Denis[14,15] to study the release of genetic information during amphibian development. He found that some DNA sites which were active in RNA synthesis at the gastrula stage continued to be active during later

development, whereas others seemed to be no longer active. As the embryo developed, stable molecules formed an increasing portion of the total population of messenger RNA. Similar studies were conducted by Church and McCarthy[12,13] on regenerating and embryonic mouse liver. Liver regeneration appeared to be partly mediated by short-lived RNA molecules, the synthesis of which commences rapidly after partial hepatectomy and ceases at various times during the regenerative process. The new species of RNA which appeared in response to partial hepatectomy were compared with those associated with rapid cell proliferation in embryonic liver. Since embryonic liver RNA was an efficient competitor for early regenerating liver RNA, it was concluded that much of the characteristic regenerating liver RNA was the result of reactivation of genes active in liver development but repressed in adult liver.

Drews and Brawerman[16] have reported that cortisol (hydrocortone) causes the appearance of new species of messenger RNA both in normal and regenerating rat liver. The administration of cortisol led to a 2- to 3-fold increase in the labeling of nuclear RNA, and affected ribosomal and messenger RNA to about the same extent. The hybridization of 20 μg of DNA with 20 μg of nuclear RNA testor from normal animals was inhibited 90% when conducted in the presence of 200 μg of nuclear RNA from normal animals, but only about 60% inhibition was observed when the testor was from cortisol treated animals. Competitor from cortisol stimulated rats inhibited hybridization with testor from normal and cortisol stimulated animals to the same extent, about 75%. No differences were observed between normal and regenerating liver 24 hours after partial hepatectomy. The 200 μg of nuclear RNA competitor is

effectively 20 times as much RNA as our 200 μg of total cellular RNA, since only about 5% of the latter is nuclear RNA, and most of the RNA that hybridizes in nuclear RNA. Later, using the same method, these authors were unable to find any such effects with growth hormone[17]. From similar studies, Gupta and Talwar[21] also concluded that while growth hormone stimulates the rate of RNA synthesis in the liver of hypophysectomized rats, it does not induce the synthesis of new RNA species to an extent detectable by their methods. Wyatt and Tata[42] reported that growth hormone and tri-iodothryonine stimulated non-hybridizing RNA more than hybridizing RNA. This was interpreted as reflecting preferential synthesis of ribosomal RNA in response to these hormones. Estrogen-mediated differentiation in chick oviduct has been studied by O'Malley and McGuire[33] who found evidence for the induction of new species of hybridizable nuclear RNA. These authors have also reported changes in hybridizable nuclear RNA during progesterone induction of the oviduct protein avidin[34]. Competitive hybridization has also been used by Neiman and Henry[32] to demonstrate the presence in human chronic lymphocytic leukemia lymphocytes of RNA species not present in normal lymphocytes. Similarly, Chiarugi[10] found hybridizable nuclear RNA species in Yoshida ascites-hepatoma and Morris 5123 hepatoma which were not present in normal rat liver.

The articles referred to above are the major reports of the detection of altered populations of cellular RNA by competitive hybridization. Now let us look at some of the details of hybridization. One should recognize the following as important questions about a hybridization system:

What kinds of RNA hybridize and to what extent? What kinds of RNA are labeled and to what extent? What levels of hybridization should one thus expect? How much RNA is required to saturate DNA gene sites? What is the difference between preincubating competitor with DNA and adding it simultaneously with the testor? How specific is hybridization?

It has been shown by McCarthy and Hoyer[30] that, as far as can be determined by hybridization, DNA from different tissues of an animal is identical. The base composition and amount of DNA per diploid nucleus from different tissues are also identical. There is no evidence of gene amplication in developed tissues of higher organisms. One can thus use DNA from brain, liver or kidney equally well for studying the hybridization characteristics of brain RNA. Virtually all RNA in animal cells is synthesized in the nuclei; a small amount is synthesized in mitochondria. A short time after pulse labeling, incorporated activity is localized in the nuclei. Thereafter, messenger, ribosomal and transfer RNA migrate out into the cytoplasm. Not all the RNA is transferred to the cytoplasm, however. Shearer and McCarthy[36] have found that approximately 80% of the RNA synthesized in the nucleus never leaves the nucleus; this RNA turns over very rapidly.

The extent to which RNA species are labeled with a pulse label depends on their turnover rates. For example, only a small fraction of the ribosomal RNA molecules, which constitute 70 or 80% of the cellular RNA and have a half-life of about 12 days in rat brain[6,23], would be labeled shortly after a pulse, while the specific activity of specific messenger RNA's with a much shorter half-life and constituting very small populations of molecules would be much higher. Those familiar

with the kinetics of brain RNA metabolism will realize that RNA isolated after 90 minutes' incorporation as in the present experiments will have a large fraction of the activity in ribosomal and transfer RNA; this can be seen from the similarity of the optical density and activity profiles when the RNA is run on sucrose density gradients. In spite of this, these species contribute negligibly to the observed hybridization, because of their low specific activity and genome representation. Other types of RNA, namely, cytoplasmic messenger and nuclear RNA, while perhaps containing less of the total activity, are responsible for almost all of the observed hybrid, because of their greater genome representation and the higher specific activities of some of their species. The greater genome representation of these types of RNA is reflected by their DNA-like base composition.

The level of hybridization one can obtain depends on what fraction of the input label is in RNA species which hybridize efficiently. With long labeling times most of the activity will be in ribosomal and transfer RNA and only very low levels of hybridization will be observed. In various cellular systems with short incorporation times, hybridization levels from 1 to 5% for RNase resistant hybrid are typically observed[16,21,42,32]; levels for total hybrid are consistently 2 to 3 times those for RNase resistant hybrid[42,31,5]. McCarthy[36] and Bondy[7], among others, do not use the RNase treatment. Some of the considerable variation between levels of hybridization reported by different groups can be attributed to differences in the way the input activity is determined; other experimental variables, of course, also contribute to this variation. Most workers have found that under the usual conditions of incubation the

maximum level of hybridization is reached after about 18 hours[38,7,12], although others find longer incubation times necessary[5]. Lowering the RNA/DNA ratio will increase the percentage of the labeled input which hybridizes, but this requires high specific activities of RNA for the detection of the hybrid. Virtually complete hybridization can be obtained in bacterial systems at very low RNA/DNA ratios[25,26]. The higher levels of hybridization at low RNA/DNA ratios are due to the more complete hybridization of ribosomal RNA and other RNA's with relatively low genome representation, some of which may be messenger RNA species. Ribosomal RNA from rat brain has been found by Stevenin et al.[38] to saturate the DNA sites coding coding for it at a RNA/DNA ratio of about 1/5. The sites for messenger RNA were found to become saturated at a RNA/DNA ratio of about 25/1. Other workers have failed to obtain saturation plateaus with nuclear RNA at RNA/DNA ratios as high as 200/1[42,10,5]. The data of Stevenin et al. indicate that .15% of the DNA genome codes for ribosomal RNA; this amounts to about 6,000 cistrons. They estimated that about 1.2% of the genome codes for messenger RNA in adult rat brain, or 50,000 to 500,000 cistrons. One can avoid the problem of differential labeling of RNA species with different turnover rates by labeling all species of RNA equally either by chronic application of label or by labeling isolated RNA with labeled dimethylsulfate[37], but usually one is interested in having only the molecules synthesized during a given short period of time labeled.

It is becoming generally recognized among workers using hybridization that the formation of hybrids does not require absolute complementarity of base sequence by the two participating strands[11,32,41].

From detailed analysis of the kinetics of DNA-DNA hybrid formation, Britten and Kohne[9] have shown that there are hundreds of thousands of copies of DNA sequences in the genome of higher animals. There appears to be some mechanism which from time to time extensively reduplicates certain segments of DNA. During evolution the repeated DNA sequences apparently change slowly and thus diverge from each other, resulting in many families of nearly identical sequences in the genome. It is estimated that these families comprise up to 30% of the base sequences in mammalian DNA. This may explain the relatively low levels of hybridization obtained with mammalian RNA. It is probable that only the products of these families of cistrons hybridize to an appreciable extent. How nearly identical sequences have to be to form hybrids is difficult to evaluate and varies with the experimental conditions. The problem of the specificity of molecular hybridization in relation to studies on higher organisms has recently been reviewed by Walker[41]. Church and McCarthy[11] suggest that the hybridization assay with mammalian nucleic acids might better be viewed as a chromatographic system in which there is a great number of different adsorption sites than as one in which specific cistrons are titrated with their own gene products. The specificity of adsorption is limited, and similar but different RNA molecules may well be indistinguishable. Thus, in competition experiments, observed differences reflect real differences although failure to discriminate does not prove identity.

The specificity of competition has also been questioned by Birnboim et al.[5]. They reported that they were unable to obtain competition when DNA loaded membranes were prehybridized with large amounts of competitor

and then hybridized with testor in another vial after the membranes had been washed. If testor was added to the competitor after prehybridization, the same amount of competition was observed as when the two were added together without any prehybridization. Riggsby and Merriam[35] found, however, that competition could be demonstrated by the prehybridization method if saturating amounts of testor were used. Using the presaturation method Chiarugi[10] obtained only about 40% saturation of active DNA sites with a RNA/DNA ratio of 500:1. In simultaneous incubation competition studies, Neiman and Henry[32] found that large amounts of heterologous animal cell RNA interupted hybrid formation in excess of the probable sequence similarity of the competing polyribonucleotides. They suggested that this nonspecific interference may have led to overestimates of the degree of similarity of the competing RNA's in some studies.

It should be kept in mind that one cannot equate rapidly labeled, DNA-like RNA with functional messenger RNA as many have done in the past. Uhrsprung et al.[40] have emphasized that at-present we have no way of identifying the biological function of the RNA that is singled out in these competition hybridization experiments.

These considerations should make it clear that it is not simple to test whether induction is involved in learning by using competitive hybridization. Only a relatively small number of brain cells need be involved in learning any particular task or fact, and the same RNA species induced in these cells could be present and turning over in large numbers in other cells which are simply maintaining their induced state. We should not expect a large effect in the hybridizability

of the total cellular RNA even if induction does take place. One should be cautious in accepting the results of Machlus and Gaito as demonstrating the induction of RNA species unique to a behavioral task, the data in the present report and those of others cast doubt upon their observed level of competition by total cellular brain RNA from trained animals. This discussion should not be construed as arguing that it will be impossible to detect new species of RNA induced through training by competitive hybridization, although at the present time it seems unlikely that this will be possible. We should not discard the idea that learning may be associated with the induction of RNA synthesis. In fact, we know that prolonged exposure of rats to an enriched environment results in "growth" in certain areas of the brain[4]. While this may not be directly related to learning, it does demonstrate an environmental (or behavioral) stimulation of macromolecular synthesis in the brain. A reproducible, demonstrable difference in the RNA populations in the brains of trained and naive animals would be highly significant, and the sensitivity of the technique could be increased over that in the present work in various ways, such as using only RNA from specific brain regions, using fractionated RNA, using larger amounts of competitor and testor, etc. Hybridization experiments with brain messenger RNA isolated from polysomes hold more promise than those using total cellular RNA. It is hoped that this discussion will be helpful to anyone interested in pursuing this technique by having pointed out some of the difficulties and ambiquities in the method.

There is at present no conclusive information concerning the role of macromolecules in learning and memory. Most biochemists and molecular biologists have rejected the idea that memories could be coded

in individual molecules, and are inclined rather to think that some sort of normal control of macromolecular synthesis is probably involved. The most obvious point of control is at the level of transcription; there is good evidence that different genes are activated during the differentiation of cells. Considering the fact that the central nervous system "pre-wiring" involved in instinctive behavior is developed from a coded sequence of bases in DNA and RNA and that inducer substances can activate specific genes, it seems quite reasonable that environmental stimuli might produce altered brain circuitry by these same molecular mechanisms. The present work has attempted to determine whether induction is involved in learning, but has failed to obtain a definitive answer to this question. Protein synthesis could also be regulated at the level of translation. The fact that so much RNA synthesized in the nucleus is broken down without leaving the nucleus has suggested to several workers still another control mechanism[11,18,39]. This may be potential messenger which is only expressed if it can escape into the cytoplasm. But it is also possible the RNA and protein synthesis have nothing directly to do with learning. One can develop models for the alteration of brain circuitry based on structural changes in synaptic membranes induced by electrical forces and maintained despite the turnover of the molecular components of the membrane.

## SUMMARY

An attempt was made to reproduce the results of a report in the literature[27] of the detection by competitive hybridization of RNA species unique to a behavioral task. The data from attempts to replicate this experiment showed no significant difference between RNA from the brains of trained and naive rats. Experiments to further characterize the hybridization system indicate that the method is probably not sufficiently sensitive to detect RNA species induced during learning if they exist. Hybridization of mammalian RNA in general is discussed with regard to the potential use of this technique in brain research for studying RNA involved in learning. The value of the technique for this purpose at present is doubtful due to complications and ambiguities with hybridization of RNA from higher organisms which are discussed.

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BIBLIOGRAPHY

1 Agranoff, B.W., Davis, R.E., and Brink, J.J., Memory fixation in the goldfish, Proc. Nat. Acad. Sci. (Wash.), 54 (1965) 788-793.

2 Attardi, G., Naono, S., Rouviére, J., Jacob, F., and Gros, F., Production of messenger RNA and regulation of protein synthesis, Cold Spring Harbor Symposium (1963) 363-372.

3 Barondes, S.H. and Cohen, H.D., Memory impairment after subcutaneous injection of acetoxycycloheximide, Science, 160 (1968) 556-557.

4 Bennett, E.L., Diamond, M.C., Krech, D., and Rosenzweig, M.R., Chemical and anatomical plasticity of brain, Science, 146 (1964) 610-619.

5 Birnboim, H.C., Pène, J.J., and Darnell, J.E., Studies on HeLa cell nuclear DNA-like RNA by RNA-DNA hybridization, Proc. nat. Acad. Sci. (Wash.), 58 (1967) 320-327.

6 Bondy, S.C., The ribonucleic acid metabolism of the brain, J. Neurochem., 13 (1966) 955-959.

7 Bondy, S.C. and Roberts, S., Hybridizable ribonucleic acid of rat brain, Biochem. J., 109 (1968) 533-541.

8. Bonner, J., Molecular biological approaches to the study of memory. In J. Gaito (Ed.), Macromolecules and Behavior, Appleton, Century and Crofts, 1966, p. 158.

9 Britten, R.J. and Kohne, D.E., Repeated sequences in DNA, Science, 161 (1968) 529-540.

10 Chiarugi, V.P., Changes in nuclear RNA in hepatomas as revealed by RNA/DNA hybridization, Biochim. Biophys. Acta, 179 (1969) 129-135.

11 Church, R.B. and McCarthy, B.J., Changes in nuclear and cytoplasmic RNA in regenerating mouse liver, Proc. nat. Acad. Sci. (Wash.), 58 (1967) 1548-1555.

12 Church, R.B. and McCarthy, B.J., Ribonucleic acid synthesis in regenerating and embryonic liver, I. The synthesis of new species of RNA during regeneration of mouse liver after partial hepatectomy, J. Mol. Biol., 23 (1967) 459-475.

13 Church, R.B. and McCarthy, B.J., Ribonucleic acid synthesis in regenerating and embryonic liver, II. The synthesis of RNA during embryonic liver development and its relationship to regenerating liver, J. Mol. Biol., 23 (1967) 477-486.

14 Denis, H., Gene expression in amphibian development, I. Validity of the method used, J. Mol. Biol., 22 (1966) 269-283.

15 Denis, H., Gene expression in amphibian development, II. Release of genetic information in growing embryos, J. Mol. Biol., 22 (1966) 285-304.

16 Drews, J. and Brawerman, G., Alteration in the nature of ribonucleic acid synthesized in rat liver during regeneration and after cortisol stimulation, J. Biol. Chem., 242 (1967) 801-808.

17 Drews, J. and Brawerman, G., Messenger RNA patterns in rat liver nuclei before and after treatment with growth hormone, Science, 156 (1967) 1385-1386.

18 Drews, J., Brawerman, G., and Morris, H.P., Nucleotide sequence

homolies in nuclear and cytoplasmic ribonucleic acid from rat liver and hepatomas, Europ. J. Biochem., 3 (1968) 284-292.

19 Flexner, L.B., Flexner, J.B., and Roberts, R.B., Memory in mice analyzed with antibiotics, Science, 155 (1967) 1377-1383.

20 Gillespie, D. and Spiegelman, S., A quantitative assay for DNA-RNA hybrids with DNA immobilized on a membrane, J. Mol. Biol., 12 (1965) 829-842.

21 Gupta, S.L. and Talwar, G.P., Effect of growth hormone on ribonucleic acid metabolism, Biochem. J., 110 (1968) 401-406.

22 Hayashi, M., Spiegelman, S., Franklin, N.C., and Luria, S.E., Separation of the RNA message transcribed in response to a specific inducer, Proc. nat. Acad. Sci. (Wash.), 49 (1963) 729-736.

23 von Hungen, K., Mahler, H.R., and Moore, W.J., Turnover of protein and ribonucleic acid in synaptic subcellular fractions from rat brain, J. Biol. Chem., 243 (1968) 1415-1423.

24 Hydén, H., Behavior, neural function and RNA. In Progress in Nucleic Acid Research and Molecular Biology, Volume 6, Academic Press, 1967, p. 186.

25 Kennell, D. and Kotoulas, A., Titration of the gene sites on DNA by DNA-RNA hybridization, I. Problems of measurement, J. Mol. Biol., 34 (1968) 71-84.

26 Kennell, D., Titration of the gene sites on DNA by DNA-RNA hybridization, II. The Escherichia coli chromosome, J. Mol. Biol., 34 (1968) 85-103.

27 Machlus, B. and Gaito, J., Detection of RNA species unique to a

behavioral task, Psychon. Sci., 10 (1968) 253-254.

28 Machlus, B. and Gaito, J., Unique RNA species developed during a shock avoidance task, Psychon. Sci., 12 (1968) 111-112.

29 Marmur, J., A procedure for the isolation of deoxyribonucleic acid from micro-organisms, J. Mol. Biol., 3, (1961) 208-218.

30 McCarthy, B.J. and Hoyer, B.H., Identity of DNA and diversity of messenger RNA molecules in normal mouse tissues, Proc. nat. Acad. Sci. (Wash.), 52 (1964) 915-922.

31 Merits, I., Schultze, W., and Overby, L.R., Ribosomal ribonucleic acid sites in chicken deoxyribonucleic acid, Arch. Biochem. Biophys., 115 (1966) 197-205.

32 Neiman, P.E. and Henry, P.H., Ribonucleic acid - deoxyribonucleic acid hybridization and hybridization - competition studies of the rapidly labeled ribonucleic acid from normal and chronic lymphocytic leukemia lymphocytes, Biochem. J., 8 (1969) 275-282.

33 O'Malley, B.W. and McGuire, W.L., Studies on the mechanism of estrogen-mediated tissue differentiation: Regulation of nuclear transcription and induction of new RNA species, Proc. nat. Acad. Sci. (Wash.), 60 (1968) 1527-1534.

34 O'Malley, B.W. and McGuire, W.L., Changes in hybridizable RNA during progesterone induction of a specific oviduct protein, Biochem. Biophys. Res. Commun., 32 (1968) 595-598.

35 Riggsby, W.S. and Merriam, V., RNA-DNA hybridization: Demonstration in a mammalian system of competition by preincubation, Science, 161 (1968) 570.

36 Shearer, R.W. and McCarthy, B.J., Evidence for ribonucleic acid

molecules restricted to the cell nucleus, Biochemistry, 6 (1967) 283-289.

37 Smith, K.D., Armstrong, J.L., and McCarthy, B.J., The introduction of radioisotopes into RNA by methylation in vitro, Biochim. Biophys. Acta, 142 (1967) 323-330.

38 Stevenin, J., Samec, J., Jacob, M., and Mandel, P., Détermination de la fraction du génome codant pour les RNA ribosomique et messagers dans le cerveau du rat adulte, J. Mol. Biol., 33 (1968) 777-793.

39 Sullivan, D., Molecular hybridization used to characterize the RNA synthesized by isolated bovine thymus nuclei, Proc. nat. Acad. Sci. (Wash.), 59 (1968) 846-853.

40 Uhrsprung, H., Smith, K.D., Sofer, W.H., and Sullivan, D.T., Assay systems for the study of gene function, Science, 160 (1968) 1075-1081.

41 Walker, P.M.B., The specificity of molecular hybridization in relation to studies on higher organisms. In Progress in Nucleic Acid Research and Molecular Biology, Volume 9, Academic Press, 1969, p. 301.

42 Wyatt, G.R. and Tata, J.R., The hybridization capacity of ribonucleic acid produced during hormone action, Biochem. J., 109 (1968) 253-258.

43 Zemp, J.W., Wilson, J.E., Schlesinger, K., Boggan, W.O., and Glassman, E., Brain function and macromolecules, I. Incorporation of uridine into RNA of mouse brain during short-term training experience, Proc. nat. Acad. Sci. (Wash.), 55 (1966) 1423-1431.

Table I

Comparison of RNA from Trained and Naive Rats as Competitor and Testor

| | Competitor | | Testor | % Inhibition ± S.D. |
|---|---|---|---|---|
| A | $RNA_{N1,2,3,4}$ (50 μg) | - | $RNA^{*}_{T4P}$ (50 μg) | 35.8 ± 4.8 |
| B | $RNA_{T1,2,3,4}$ (50 μg) | - | $RNA^{*}_{T4P}$ (50 μg) | 38.0 ± 2.3 |
| A | $RNA_{N1,2,3,4}$ (200 μg) | - | $RNA^{*}_{T4P}$ (50 μg) | 54.0 ± 8.3 |
| B | $RNA_{T1,2,3,4}$ (200 μg) | - | $RNA^{*}_{T4P}$ (50 μg) | 58.5 ± 3.4 |
| A | $RNA_{N1,2,3,4}$ (50 μg) | - | $RNA^{*}_{T1,2,3,4}$ (50 μg) | 32.7 ± 4.9 |
| C | $RNA_{N1,2,3,4}$ (50 μg) | - | $RNA^{*}_{N1,2,3,4}$ (50 μg) | 36.8 ± 3.1 |

The first two pairs of data compare 50 and 200 μg of competitor from trained and naive rats for their efficiencies in inhibiting subsequent hybridization with testor RNA from trained rats. The numerical subscripts refer to RNA isolated from individual rats; label is indicated by *. Each percent inhibition is the average of four with its standard deviation. The third pair compares trained and naive testor after hybridization with naive competitor. Each percent inhibition is from sixteen determinations.

Table II

Possible Combinations of Competitor and Testor RNA

| | Competitor | Testor | Examined |
|---|---|---|---|
| A | naive | trained | Machlus and Gaito<br>this work: Table I |
| B | trained | trained | this work: Table I, Figure 2 |
| C | naive | naive | this work: Table I |
| D | trained | naive | Machlus and Gaito<br>this work: Figure 2 |

## Figure Captions

Figure 1 Learning curves for trained rats. Escape latency is the time taken for the animal on each trial to run into the lighted box after being placed in the dark box. Average of 4 unlabeled rats, ○; average of 6 labeled rats, □.

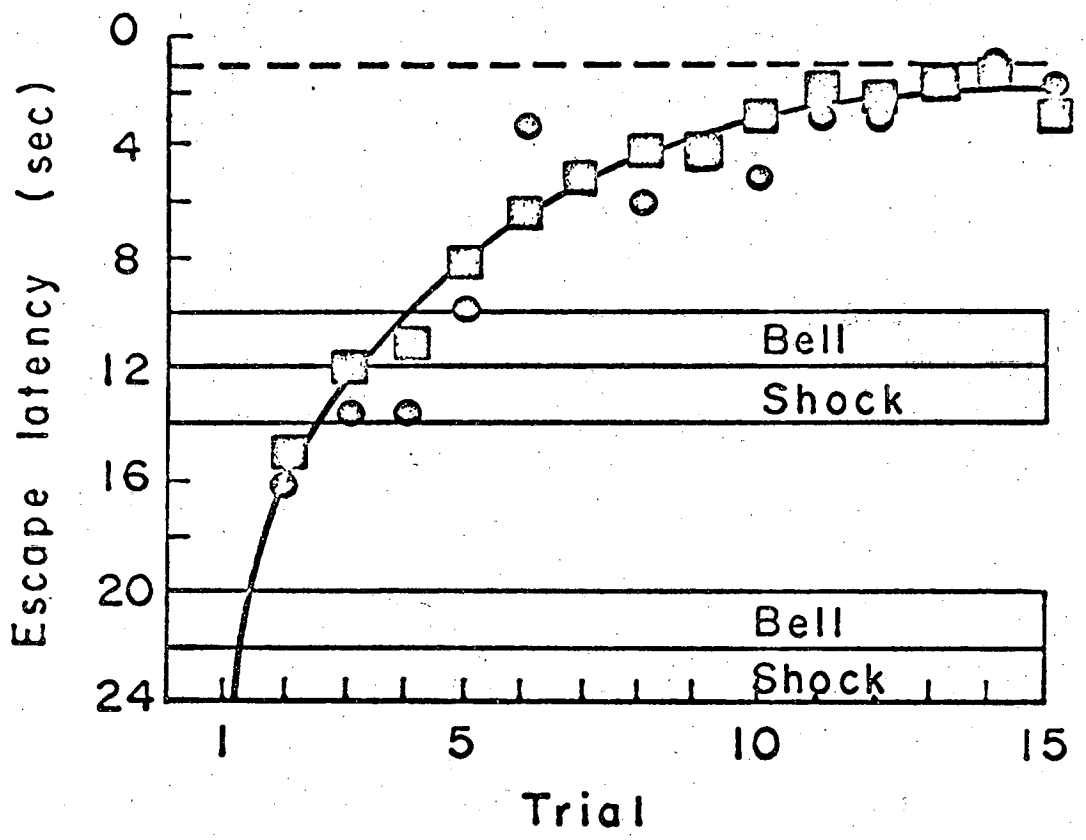
Figure 2 Nitrocellulose membranes ~~xxxx~~ loaded with 50$\mu$g of DNA were hybridized for 12 hours with various amounts of unlabeled competitor RNA, after which 50$\mu$g of labeled testor RNA was added and the hybridization continued for 12 more hours. The results are expressed as percent of control, the level of hybridization of testor when no competitor was added. In the first experiment, in which RNase resistant hybrid was determined, the competitor was pooled RNA from the brains of 4 trained rats, and the testor was pooled RNA from the brains of 4 naive rats, □. In the second experiment both RNase resistant, ◐, and total hybrid, ○, were determined; the competitor was the same as in the first experiment, and the testor was pooled RNA from the brains of 4 trained rats.

Figure 3 The relationship between the amount of labeled RNA input and the amount of RNase resistant, □, and total hybrid, ○, formed after 12 hours of hybridization. The specific activity of the RNA was 30 cpm/$\mu$g. Membranes were loaded with 50$\mu$g of DNA.

Figure 4 The effect of the duration of hybridization on the percentage of input forming RNase resistant, ▩ , and total hybrid, O. The input was 50$\mu$g of RNA, 30 cpm/$\mu$g. Membranes were loaded with 50 g of DNA.

Fig 1

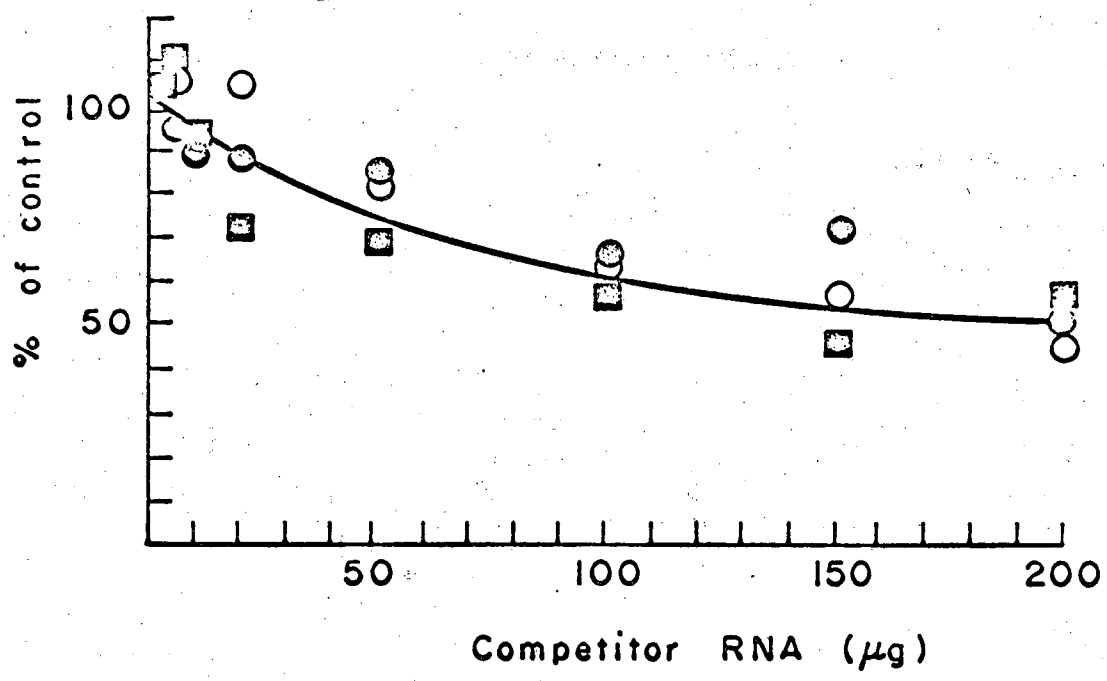





XBL696-2994

Fig 2

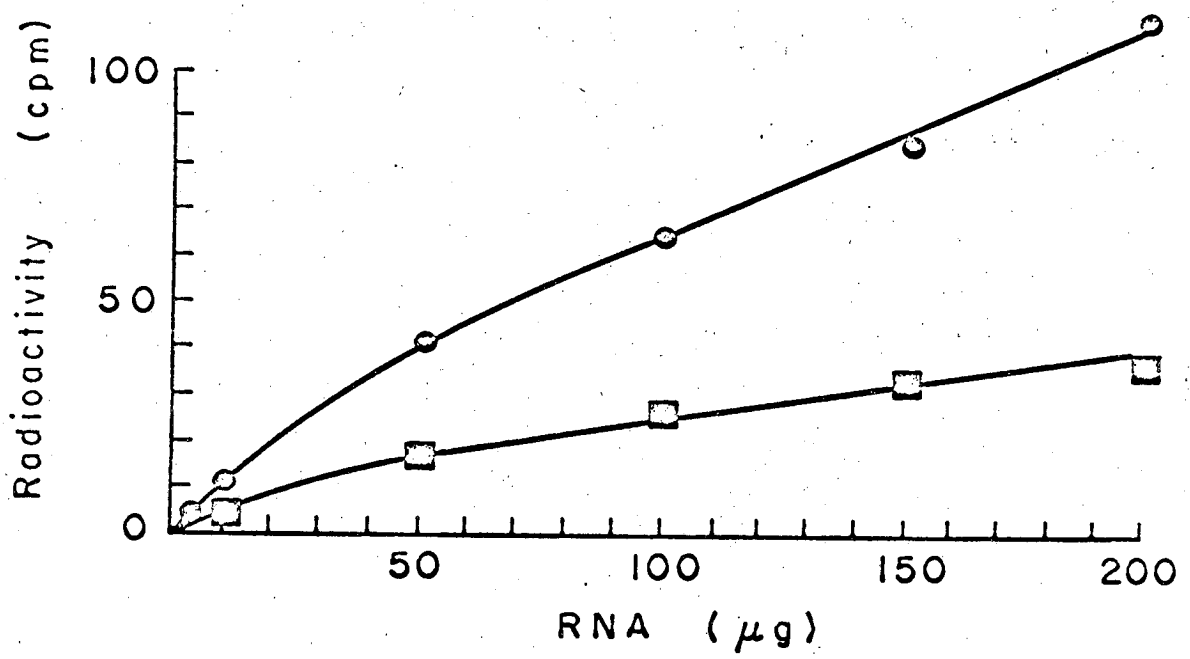





XBL696-2995

Fig 3

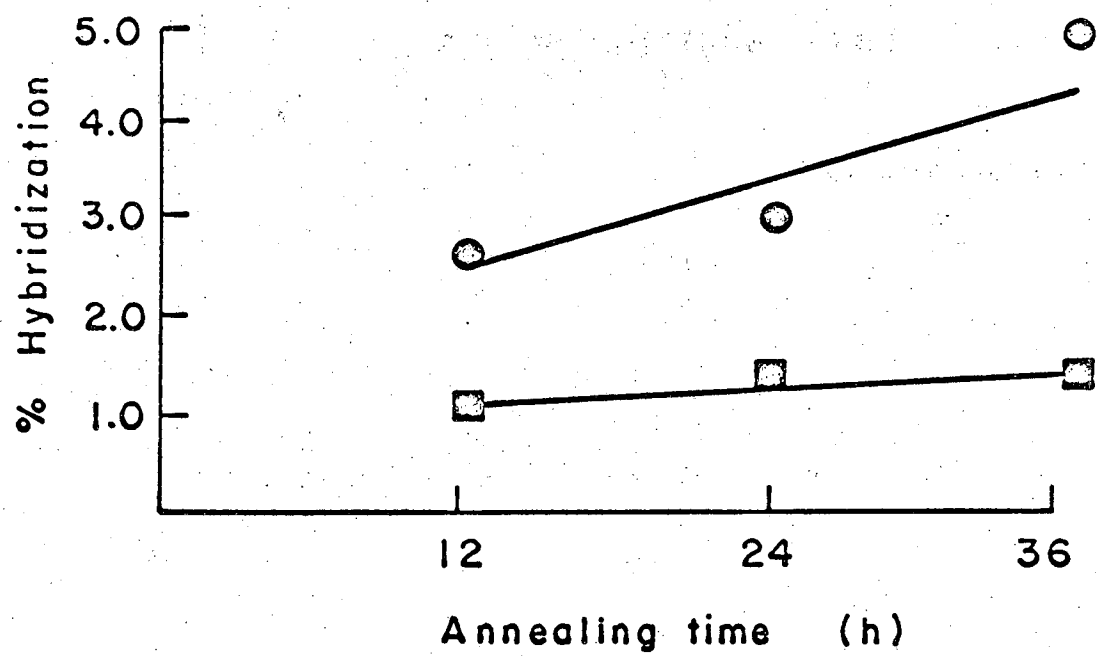





XBL696-2993

Fig 4



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